Acaricidal activity of Essential oil Based Nanoformulations Against Cattle Tick *Rhipicephalus microplus*

SYNOPSIS

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INTRODUCTION

Ectoparasites are a major threat for the livestock with about 80% of world's cattle population infested with ticks (FAO, 2004). India accounts for a significant share of the world's livestock resources and the damage caused by ticks and tick-borne diseases (TTBDs) to livestock is considered very high (Ghosh *et al*. 2006). The cattle tick, *Rhipicephalus microplus* is a monogenetic ectoparasite that is haematophagus and feeds on bovines. It is considered one of the most harmful cattle parasite in sub-tropical areas of the world as it causes great loss to the cattle industry directly or indirectly through, blood loss, lesions, reduced weight and milk production and through biological transmission of tick-borne pathogens such as *Babesia* spp. and *Anaplasma marginale* (Jonsson, 2006)

In the past, management of ticks heavily relied on the excessive use of synthetic chemical acaricides as they are fast acting however, the use of chemical acaricides is costly and harmful for environment, humans and livestock. Moreover, many reports are currently available on the development of tick resistance from different parts of India (Ahanger *et al*. 2015). Conventional approaches such as Pasteur management, spraying insecticides are not able to fulfill the tick control requirements. This has promoted scientists to search for new controlling strategies for these notorious ectoparasites.

The use of herbal based preparations attracted many researchers for controlling this ectoparasite due to their biodegradability, target efficiency and cost effectiveness. Essential Oils (EOs) are the plant secretion derived from various parts of the plant such as leaf, flower, bud, stem, etc. EOs are the volatile compounds, having strong aroma and are the by-products of the plant metabolism and have been used for many biological properties such as antibacterial, fungicidal, insecticidal etc. They have also been tested against ticks recently (Kheirabadi *et al*., 2011; Pazinato *et al*., 2016).

Now a day's nanotechnology emerged as a booming field with wider application in various fields that has attracted various researchers due to unconventional physical, chemical, and biological characterstics of nanopreparations.There are two main approaches suggested for the preparation of nanoformulations. First,

the low energy emulsification method in which the chemical energy is stored in the surfactant to be used as the emulsifiers. Second, the high energy emulsification method that needs mechanical stirring, homogenization and ultrasonication.

For effective management of ticks an integrated approach is warranted which may not only control them but also prevent reinfestation of cattle with ticks. Use of repellents may be one such strategy hence, development of herbal repellent nanoformulations and their controlled release is an alternative to tick management.

It is evident from literature survey that only essential oils have been tested in recent past for their acaricidal activity however, no reports exists till date on the enhanced activity of these oils through nanoformulations. Therefore, the present study would focus on the synthesis and characterization of essential oil based nanoformulations and their acaricidal activity against *Rhipicephalus microplus*. Further, any possible toxic effects of these formulations would also be tested on HEK 293 mammalian cell lines so as to develop the safer acaricide for the livestock.

LITERATURE REVIEW

Rhipicephalus microplus 'the tropical cattle tick' is an economically important ectoparasite and major threat to the cattle as it spreads wide number of diseases like babesiosis, rocky spotted mountain fever, tick paralysis, anaplasmosis etc. Now a day's there is a growing concern about the use of herbal preparations for controlling the ticks due to their cost effectiveness, target specificity and lack of adverse effects. A number of studies have been conducted to screen the acaricidal potential of plant extracts and are reviewed here.

Vatsya and Dass (2004) studied *in vitro* acaricidal property of few herbal plants against *Boophilus microplus* while, Pathak *et al*. (2004) performed *in vivo* study of neem leaves and barks, nochi leaves, vashamber rhizome and punzu leaves in methanolic extracts against Ixodid ticks in small ruminants. Choudhary *et al*. (2004) investigated *in vitro* study of *Nicotiana tobaccum* in aqueous medium against *R. haemaphysaloides* in the same year.

Wang *et al.* (2007) formulated and characterized the oil in water nanoemulsions against pest with low energy approach for nanoformulation. Experiments were conducted to study the acaricidal action of neem extracts against *B. microplus* by Srivastava *et al*. (2008). Rony *et al.* (2010) studied the epidemiology of ectoparasitic infestations in cattle at Bahwal forest area, Gazipur. The prevalence rate was highest in case of *B. microplus* followed by *R. sanguineus, Linognathus vituli, Haematopinus euysternus, Hemaphysalis bispinosa and Damalinia bovis*. He also found that infestation rate was higher in animals reared under free ranging system. Essential oils extracted from *Hesperozygis ringens* (Ribeiro *et al*., 2010) and essential oils of *Tetradenia riparia* (Gazim *et al*., 2011) were tested for acaricidal potential to control *R. microplus.* Kumar *et al.* (2011) investigated *in vitro* and *in vivo* acaricidal activity of some indigenous plants under organized and farmer flock. Chagas *et al.* (2011) studied *in vitro* activity of *Artemisia annua* extracts against *R. microplus* and he found that alone *in vitro* is not adequate for effective evaluation of the action of *A. annua* on *R. microplus.* Giglioti *et al.* (2011) also studied acaricidial activity of neem seed

extracts with known azadirachtin concentrations against *R. microplus*. He found in his study that neem seeds contain high concentrations of AZA and limonoids compounds which have adverse effect on the reproduction of *R. microplus* females. Kheirabadi *et al*. (2011) studied the *in vitro* acaricidal properties of essential oils derived from *Zataria multiflora* and *A. annua* in order to control cattle ticks. He found that essential oils applied at more than 20 and 60 µl/ cm^3 caused 100% failure in egg laying capacity of engorged female ticks and the mortality rate was dose dependent in ticks. Ribeiro *et al.* (2011) evaluated the efficacy of acaricides used to control *R. microplus* in dairy cattle in South west Brazil. Elango and Rahuman (2011) evaluated the various medicinal plant extracts against ticks and fluke for anti-parasitic activity. Out of various medicinal plants *T. erecta, A. marmelos and A. paniculata* found to be good promising biocontrol aspirant for use against *R. microplus, P.cervi and H. bispinosa.* Patel *et al.* (2012) studied the prevalence and seasonal variation in Ixodid ticks in relation to different month, seasons, age, sites of attachment and its identification in Mathura district, Uttar Pradesh. Ghosh *et al*. (2013) evaluated the leaf extracts of *Riccinus communis* against Pyrethroids (PT) and Organophosphate (OP) resistant in *B. microplus*. Further, Nantarat *et al*. (2015) investigated the optimization, characterization and stability of essential oils blend nanoemulsions for anti-tyrosinase activity by low energy approach PIC technique using coconut carrier oil, PEG 40 and sorbitan monoleate. This nanoemulsion blend showed good inhibitory effect on *Agaricus* tyrosinase.

Recently, Ahanger *et al.* (2015) studied the detection of deltamethrin resistance in field populations of *R. microplus* in Jammu and Kashmir. Relative acaricidal bioefficacy of Adhatoda vasica's root against *R. microplus* (Acari: Ixodidae) in methanolic, hexane or petroleum ether extract was demonstrated by Kishore *et al*. (2015). Campos *et al.* (2015) used vetiver essential oil (Family-Poaceae) for the acaricidal action against two tick species *R. microplus* and *Amblyomma cajennense*. Ghosh *et al*. (2016) characterized the acaricide resistance in tick species in Rajasthan region. Pazinato *et al*. (2016) also investigated the *in vitro* effect of seven essential oils on the reproduction of the cattle tick *R. microplus*. Geranium oil showed the 100% repellency against Ixodes nymphs. Silvalima *et al*. (2016)

studied the repellency effect of *Lippia alba* essential oil and major constituent against cattle tick *R. microplus*. Shah *et al*. (2016) reviewed the use of nanotechnology approach for the development of insecticidial formulations

OBJECTIVES

- Synthesis and characterization of the essential Oil based nanoformulations
- Acaricidal efficacy study of nanoformulations against cattle tick *Rhipicephalus microplus*
- Toxicity assessment of the essential oil based nanoformulations in HEK 293 mammalian cell lines.

MATERIALS AND METHODS

A. MATERIALS:-

Experimental animal: *Rhipicephalus microplus*

Rhipicephalus microplus belongs to family Ixodidae (hard tick) and a haematophagus ectoparasite. *R. microplus* is an economically important tick that parasitizes a variety of livestock species and it is known as the cattle tick or southern cattle tick. It has been recorded on cattle, buffalo, horses, donkeys, goats, sheep, deer, pigs, dogs and some wild animals.

Taxonomic Position:-

Kingdom: Animalia

Phylum: Arthropoda

Class: Arachnida

Order: Acarina

Family: Ixodidae

Genus: *Rhipicephalus*

Species: *microplus*

Ticks cause severe damage to cattle rearing industry that leads to weight loss**,** blood loss and even death in some cases.They are widely distributed arachnids having 18 genera and 900 species all over the world. They are especially found in the regions which are humid and warmer. Tick adults are ovoid or pear shaped body structure. Hard ticks have dorsal shield and their mouth parts are protrude forward when they are seen from front. A female can lay upto 1000 eggs in single batch. Hatching of eggs can be in 2 to 20 days later depending upon the weather conditions. They complete their life cycle in four stages i.e. egg, larva, nymph and adult. Each stage requires blood for metamorphosis. Their life span is of 2 month-3 years.

Essential Oils:

Essential Oils (EOs) are the plant secretion derived from various parts of the plant such as leaf, flower, bud, stem, etc. extracted by hydro- and steam distillation. EOs are the volatile compounds, having strong aroma and are the by-products of the plant metabolism. Most EOs comprise of monoterpens, phenols, sesquiterpenes.The aromatic characterstics of EOs provide various characterstics functions for the plants such as attracting and repelling insects. Therefore, they have been used for many biological properties such as antibacterial, fungicidal, virucidal, anti- oxidant, insecticidal etc.

In the present study, EOs obtained through steam distillation such as **Lemon grass oil, Neem oil, *Eucalyptus* oil, *Calendula* oil, Camphor oil** would be used for the preparation of nanoformulations for evaluating acaricidal properties against *R. microplus.*

B. <u>METHODOLOGY</u>:

Collection of ticks

Collection of engorged females will be done from cattle of Agra which would be transported to the laboratory in plastic boxes having holes. The specimens will be identified by using standard tick identification keys. Ticks will be washed with distilled water before conducting the bioassays.

Synthesis of Nanoformulations

Different nanoformulations will be prepared and then acaricidal property would be evaluated against *R. microplus*. Essential oil based nanoformulations would be synthesized by using high energy approach i.e. Ultrasonication. In this, steam distilled extracted essential oils such as **Lemon grass oil, Neem oil, *Eucalyptus* oil, *Calendula* oil, Camphor oil** along with polyethylene glycol 400 and deionised water will be used for the development of nanoformulations. PEG 400 will be used as surfactant as it is strongly hydrophilic, non- toxic, also miscible in aromatic compounds, pH stable and compatible with other

ingredients (Nantarat *et al.* 2015). Essential oils and surfactant or co-surfactant will be mixed in different proportions to optimize the protocol for the nanodroplet size.

Characterization of Essential oil based Nanoformulations:

The resultant nanoformulations will be analyzed with the help of Transmission Electron Microscopy (TEM) for the morphological study, Zeta potential for the measurement of surface charge, pH, viscosity of nanoformulation, droplet size and nano droplet size distribution will also be analyzed by DLS method, stability of nanoformulation will also be checked by freeze thaw cycles, at different temperatures etc. as these parameters are necessary for any characterization of nanoformulations. DLS Characterization of nanoformulations will be carried at Department of Chemistry, DEI and TEM will be done at AIIMS, New Delhi.

Acaricidal efficacy study:

Adult Immersion test:

The ticks will be collected from natural infested cattle. The obtained ticks will be divided in groups with five engorged females would be placed in each group. These ticks would be weighed to make homogeneous group for study. They would be immersed in various concentrations of test solutions, positive (DEET) and negative controls for 3-5 mins. Ticks would be removed and left for drying on tissue paper and transferred to the petridish containing whatman no. 1 filter paper. They would be observed after **24 h for acaricidal activity and subsequent number of days till the egg laying for reproductive activity. The positive and negative controls would also be run in parallel to set up with DEET or Permethrin, surfactant and distilled water following similar protocol** (Drummond *et al*., 1973).

Reproductive index:

The ticks survived after exposing to different concentrations of nanoformulations would be used subsequently for generating the data on effect of most effective concentration on reproductive index (RI = egg mass/engorged tick weight) (Ghosh *et al.,* 2013). Survived ticks would be individually weighed and the eggs laid by each tick will be weighed separately.

The optimum dose (concentration) of most effective nanoformulation will be determined on the basis of highest percent mortality and lower RI.

Larval packet assay:

For this test Whatman filter paper NO. 1 in parallelogram shape (5.5cm x 5 cm) and impregnated with various concentrations of test and control solution and dried for about half an hour in incubator at 37˚C. Treated papers will be folded into packets and a fixed number of larvae will be placed which will be sealed with cellotape. The packets after placing in dessicator would be kept in BOD incubator set at 28±1˚C and 85 ± 5% RH. After 24 hours, larval mortality will be checked (Kumar *et al.,* 2016).

Repellency test:

Test would be conducted by following the protocol of Mawela (2008) in which glass rod will be vertically fixed in a beaker. On the top of the glass rod, impregnated filter paper will be pasted having 100µl of the test compound i.e. nanoformulations, DEET and DMSO and non-impregnated filter paper below it. Before starting the bioassay, the filter paper that is impregnated or non-impregnated will be left for air drying at room temperature for 30 s. All compounds would be dissolved in 3% dimethyl sulphoxide (DMSO) where DMSO alone will serve as negative control, DEET would serve as positive control and rest of test concentrations from nanoformulations will be used in a similar manner. Repellency would be calculated according to the following formula :

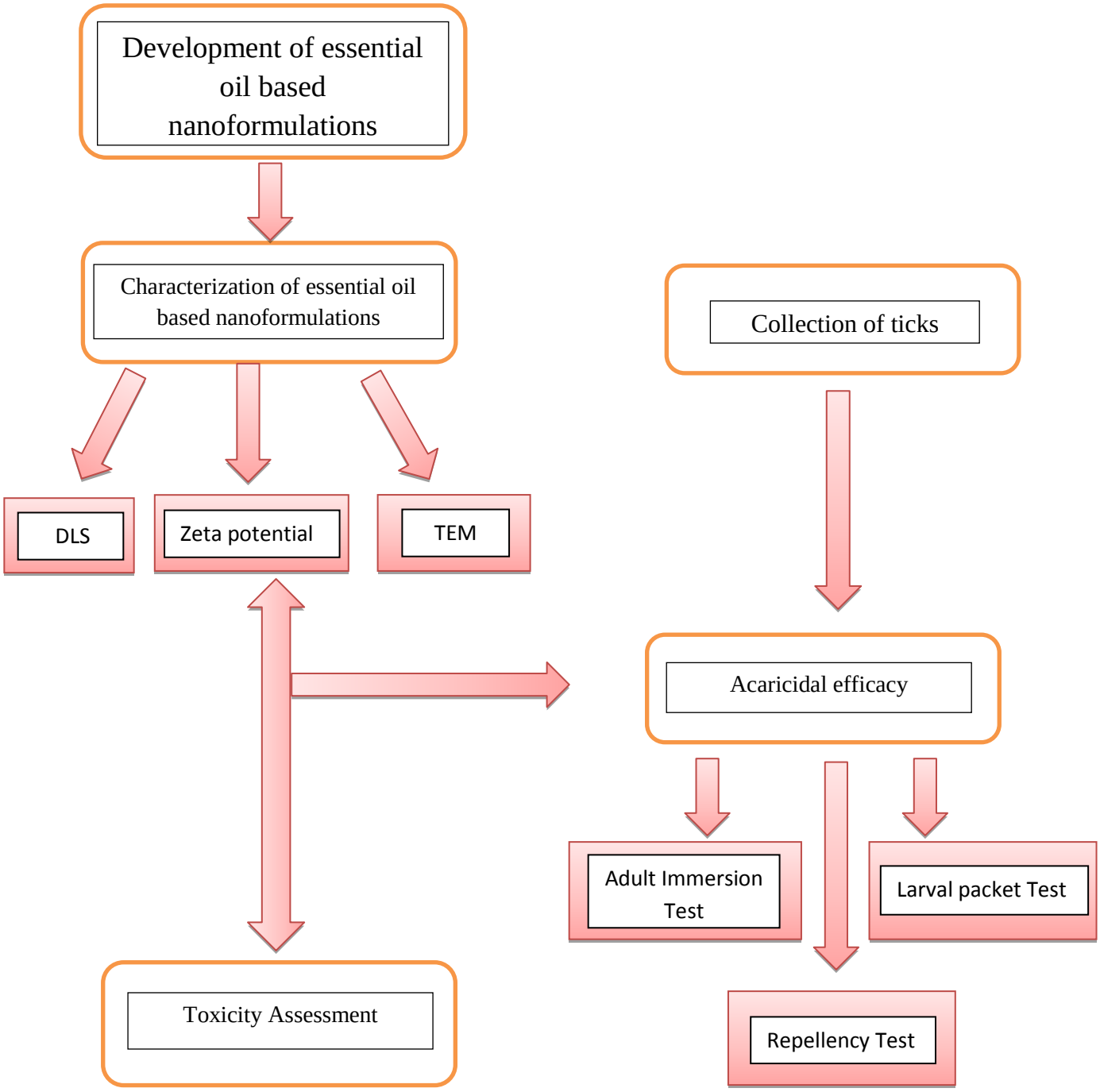
(No. of larvae on non impregnated filter paper ×100)/ (No. of larvae on non impregnated filter paper + No. of larvae on impregnated filter paper).

Statistical analysis: The data will be presented as mean±SE and differences in mean values of data among the groups will be analyzed by Student's t test. 50% mortality will be indicated by EC_{50} and calculated through SPSS version 19.0.

Toxicity assessment

The toxicity assessment will be done for developing safer acaricidal nanoformulations. For this purpose, toxic effects of these formulations, if any, would be evaluated following basic protocol of MTT Assay described by Mosmann (1983) on mammalian cell lines, Human embryonic kidney cells 293, also known as HEK 293.Viability of cells will be checked by the use of MTT (3-(4,5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) for assessment of cytotoxicity. MTT is a yellow coloured dye, which is reduced into violet colour formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells. The cells would be cultured in 96-well microtiter plates using DMEM along with 10% FBS and 1% Penicillin streptomycin as a culture medium followed by an overnight incubation at 37^{o} C and will treated with varying concentrations of different formulations for 48 hours. At the end of the treatment period, 10µl of MTT will be added to each well and the plates will be incubated for 3 h in a dark chamber and supernatant from each well would be aspirated. Finally, 100µl of DMSO will be added to dissolve the formazan crystals. Absorbance will be recorded at 595 nm with microplate reader. This part of the study would be carried out at ICGEB, New Delhi.

EXPERIMENTAL DESIGN



PLAN OF WORK

PHASE 1

- Synthesis of essential oil based Nanoformulations
- Characterization of Nanoformulations through: TEM, Zeta potential, DLS etc.

PHASE 2

- Acaricidal efficacy study against *Rhipicephalus microplus*- Bioassays
  - Adult immersion assay
  - Larval packet assay
  - Repellency test

PHASE 3

- Toxicity assessment of various nanoformulations in HEK 293 mammalian cell lines through:

  MTT assay

REFERENCES

- Ahanger R.R., Godara R., Katoch R., Yadav A., Bhutyal A.D.S., Katoch M., Singh N.K., Bader M.A. 2015. Deltamethrin resistance in field populations of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) in Jammu and Kashmir, India. Exp. Appl. Acarol. 67: 467-475.
- Campos R., Lima C., Oliveira A., Araujo A., Blank A., Alves P., Lima R., 2015. Acaricidal properties of vetiver essential oil from *Chrysopogon zizanioides* (Poaceae) against the tick species *Amblyomma cajennense* and *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). Vet. Parasitol. 212(3–4): 324-330.
- Chagas A., Georgetti C.S., Carcalho C., Oliveira M., Rodrigues R., Foglio M., Magalhaes P. 2011. *In vitro* activity of *Artemisia annua* L (Asteraceae) Extracts against *Rhipicephalus (Boophilus) microplus*. Rev. Bras. Parasito. Vet. Jabo. 20: 31-35.
- Choudhary R.K., Vasanthi C., 2004. *In vitro* effect *Nicotiana tobacum* aqueous extract on *Rhipicephalus haemaphysaloide*s ticks. Ind. J. Animal Sc. 74: 730-731.
- Drummond R.O., Ernst S.E., Trevino J.L., Gladney W.J., Graham O.H. 1973. *Boophilus annulatus* and *Boophilus microplus*: Laboratory test of insecticides. J. Eco. Entomol. 66: 130-33.
- Elango G, Rahuman A.A. 2011. Evaluation of medicinal plant extracts against ticks and fluke. Parasito Res. 108: 513-519.
- FAO, 2004. Resistance Management and Integrated Parasite control in Ruminants—Guidelines, Module I- ticks: Acaricide Resistance: Diagnosis, Management and Prevention. Food and Agriculture Organization, Animal Production and Health Division. Rome, 25–77.
- Gazim Z.C., Demarchi I.G., Lonardoni M.V., Amorim A.C., Hovell A.M., Rezende C.M., Ferreira G.A., De Lima E.L., De Cosmos F.A., Cortez D.A. 2011. Acaricidal activity of the

essential oil from *Tetradenia riparia* (Lamiaceae) on the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) Exp. Parasitol. 129 (2): 175-180.

- Ghosh S., Sharma A., Nagar G., Rawat S., Tiwari S., Kumar R., Dhakad M., Sharma R., Saxana R., Mehraniya R., Singh R., Jain D., Rai A., Ray D. 2016. Characterization of acaricide resistance in tick isolates collected from Rajasthan, India. Ind. J. Ani. Sc. 86 (1): 14-23
- Ghosh S., Tiwari S.S., Srivastava S., Sharma A.K., Kumar S., Ray D.D., Rawat A.K. 2013. Acaricidal properties of *Ricinus.communis* leaf extracts against organophosphate and pyrethroids resistant *Rhipicephalus (Boophilus) microplus*. Vet. Parasitol. 18: 192 (1-3): 259-267
- Ghosh S., Azhahianambi P., de la Fuente P. 2006. Control of ticks of ruminants, with special emphasis on livestock farming system in India: present and future possibilities for integrated control—a review. Exp. Appl. Acrol. 40:49–66
- Giglioti R., Oliveira H., Forim M.R., Chagas A.C.S., Ferrezini J., Brito L.G., Falcoski T., Albuquerque 2011. *In vitro* acaricidal activity of neem (*Azadirachta indica*) seed extracts with known azadirachtin concentrations against *Rhipicephalus microplus.* Vet. Parasitol 181: 309-315
- Jonsson N.N. 2006. The productivity effects of cattle tick (*Boophilus microplus*) infestation on cattle, with particular reference to *Bos indicus* cattle and their crosses. Vet. Parasitol. 137: 1–10.
- Kheirabadi K.H., Silva J.A. 2011. *In vitro* assessment of the acaricidal properties of *Artemisia annua* and *Zataria multiflora* essential oils to control cattle ticks. Iran. J. Parasitol. V 6 pp 58-65.
- Kishore V., Srivastava C.N., Kumar S., Mohan L., 2015. Relative acaricidal bioefficacy of certain root extract of *Adhatoda vasica* against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). Int. J. Pharma. Res. Bio. Sc. 4: 224-235.
- Kumar A., Singh S., Mahour K., Vihan V.S. 2011. *In vitro* and *in vivo* acaricidal activity of some indigenous plants under organized and farmer flock. Pharmacologyonline 3: 361-369.

- Kumar S., Sharma A. K., Nagar G., Rawat S. S., Tiwari S. S., Kumar, R. *et al*. (2016). Characterization of acaricide resistance in tick isolates collected from Rajasthan, India. Ind. J. Ani. Sc., 86(1), 14-23.
- Mawela K.G. 2008. The toxicity repellent properties of plant extracts used in ethnoveterinary medicine to control ticks. Dissertation. University of Pretoria, Pretoria.
- Mossmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65:55-63.
- Nantarat T., Chansakaow S., Leelapornpisid P. 2015. Optimization, characterization and stability of essential oils blend loaded nanoemulsions by PIC technique for Anti tyrosinase activity. Int. J. Pharm Sci. 7: 308-312.
- Patel G., Shanker D., Jaiswal A.K., Sudant V., Verma S.K. 2012. Prevalence and seasonal variation in ixodid ticks on cattle of Mathura district, Uttar Pradesh. J. Parasito Dis. 37: 173-176.
- Pathak D., Mathur V.C. 2004. *In vitro* effect of indeginous plant extracts on Ixodid ticks of small ruminants. Ind. J. Animal Sc. 74(6) 616-617.
- Pazinato R.,Volpata A., Baldissera M.D., Santos R.C.V., Baretta D., Vaucher R.A., Giongo J.L., Boligon A.A., Stefani L.M., Silva A.S.D. 2016. *In vitro* effect of seven essential oils on the reproduction of the cattle tick *Rhipicephalus microplus*. J. Adv. Res. 1030-1034.
- Ribeiro V.L., Dos Santos J.C., Bordignon S.A., Ape I.M.A., Henriques A.T., Von Poser G.L. 2010. Acaricidal properties of the essential oil from *Hesperozygis ringens* (Lamiaceae) on the cattle tick *Rhipicephalus (Boophilus) microplus*. Biores. Tecnol. 7: 2506-2509.
- Ribeiro E., Brito L., Barbieri F., Rocha R., Oliveira M. 2011. Evaluation of the efficacy of acaricides used to control the cattle tick, *Rhipicephalus microplus*, in dairy herds raised in the Brzilian Southwestern Amazon. Vet. Med. Int. doi:10.4061/2011/806093.
- Rony S.A., Mondal M.M.H., Begum N., Islam M.A., Affroze S. 2010. Epidemiology of ectoparasitic infestations in cattle at Bahwal forest area, Gazipur. Bangl. J. Vet. Med. 8(1): 27-33.

- Shah M.A., Wani S., Khan A., 2016. Nanotechnology and insecticidal formulation. J. Food Bioengg. Nanoprocess. 3: 285-310.
- Silvalima A., Carvalho J.F., Peixoto M.G., Blank A.F., Borges L.M.F., Junior L.M. 2016. Assessment of the repellent effect of *Lippia alba* essential oil and major monoterpens on the cattle tick *Rhipicephalus microplus.* Med. Vet. Entomo. 30: 73-77.
- Srivastava R., Ghosh S., Mandal D.B., Azhahianambi P., Singhal P.S., Pandey N.N., Swarup D. 2008. Efficacy of *Azadirachta indica* extracts against *Boophilus microplus*. Parasitol. Res.104: 149-153.
- Vatsya S. and Dass S.S. 2004. Acaricidial effect on the biological activities of female *Boophilus microplus.* J. Vet. Parasitol. 18(1): 91-92.
- Wang L., Li X., Zhang G., Dong J., Eastoe J. 2007. Oil-in-water nanoemulsions for pesticide formulations. J. Coll. Int. Sc. 314: 230-235.